IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Hidenori Yamada et al.

Serial No.: 10/671,881 Art Unit: 5366

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Title : TEST KIT FOR INTRACELLULAR INTRODUCTION OF PROTEIN AND/OR PEPTIDE AND METHOD OF INTRACELLULAR INTRODUCTION

DECLARATION UNDER RULE 1.132

Honorable Commissioner of Patents and Trademarks,
Washington, D.C. 20231

Sir:

I, Junichiro Futami, a citizen of Japan and having postal mailing address of Shimizu, 2-1-31 F-conforce 203, Okayama 703-8243 Japan, declare and say that:

March 1999, I was graduated from Department of Bioscience and Biotechnology, Graduate School of Natural Science and Technology, Okayama University, and received a Philosophiae Doctor in Bioscience and Biotechnology;

From October 2005, up till the present, I have been employed as a lecturer by Department of Medical and Bioengineering Science, Graduate School of Natural Science and Technology, Okayama University, and engaged in the works of research and development for Protein Engineering and Biochemistry;

I am one of the inventors of the above-identified application am familiar with the subject matter thereof;

I have read the Office Action mailed and the references cited therein and am familiar with the subject matter thereof;

I respectfully submit herewith my exact report thereon;

(1) Purpose

To verify that reduction in introduction efficiency is attributed to more than 3000 of the number average molecular weight (Mn) of Polyethyleneimine (PEI)

(2) Brief Summary Of Experiment

PEIs having various Mn(Mn=600, 1200, 1800, 3000 and 5000) were binded with avidin, and further binded with biotinylated GFP as a fluorescent protein to form conjugates. These conjugates were introduced into cells. In the cells into which the conjugated was introduced, the nucleuses of all the cells were stained with Hoechst33342, and the nucleuses of dead cells were stained with Propidium Iodidl (PI).

The introduction efficiency of the GFP was evaluated base on images of confocal laser microscope. The dead cells were evaluated based on the PI staining images. Using a fluorescent microscope, the number of all the cells was measured based on the Hoechst33342 staining images, and the dead cells identified based on the PI staining images were evaluated.

(3) Experiment description

<Sample>

Polyethyleneimine (product of NIPPON SHOKUBAI CO., LTD.)
Mn= 600, 1200, 1800, 3000, 5000

Chicken Avidin (product of Nacalai Tesque CO., LTD.)

Propidium Iodide (product of Wako Pure Chemical Industries, Ltd.)

Hoechst33342 (product of DOJINDO LABORATORIES)

GFP (in house; prepared in Escherichia coli using EGFP-

gene produced by Clontech corp.)
HeLaS3 cell (product of ATCC)

<Principal experiments>
Confocal laser microscope (Carl Zeiss LSM-510 META)
Fluorescent microscope (Carl Zeiss Axiovert200M)
Others (generally used apparatuses)

<Experimental conditions>
1. HeLaS3 cell was seeded in a 12 well plate and the cell density was adjusted to about 30% confluent.
2. *Modified Avidin (final concentration of 150 nM (tetramer of the active structure was defined as 1 molecule) and Biotin-SS-GFP (final concentration of 100 nM) were mixed in 0.5 mL of DMEM+10%FBS culture medium and left for 10 minutes at a room temperature to form a conjugate.
   "*Modified Avidin" is prepared by binding PEI (Mn= 600, 1200, 1800, 3000 and 5000) and Chicken Avidin, respectively.
3. The culture supernant of the cell of 1 was removed and replaced with the solution of 2.
4. Culture 3 for 3 hours at 37°C.
5. The culture medium was removed from 4 and treatment was performed for 20 minutes in PBS+1%BSA containing 1μg/mL of Propidium Iodido (PI) and 200 ng/mL of Hoechst33342.
6. Using a fluorescent microscope, the nucleuses of the living or dead cells were visualized with Hoechst 33342 (blue) and the dead cells were visualized with PI (red).
7. The green fluorescence of the GFP was visualized using a confocal laser microscope (excitation light: 488nm).

<Results>
Fig.1 shows views by fluorescent microscope.

Fig.2 shows views of native Avidine by confocal laser microscope.
Fig.3 shows views of Avidine-PEI600 by confocal laser microscope.
Fig.4 shows views of Avidine-PEI1200 by confocal laser microscope.
Fig.5 shows views of Avidine-PEI1800 by confocal laser microscope.
Fig.6 shows views of Avidine-PEI3000 by confocal laser microscope.
Fig.7 shows views of Avidine-PEI5000 by confocal laser microscope.

In the figures, the green fluorescence of the GFP shows fluorescence transmitted Band-pass-Filter (BP505-530), and the red fluorescence of PI shows fluorescence transmitted Interference-Filter (LP585). Additionally, "Dig" in Fig.2 to 7 shows images taken by Differential Interference Contrast.

<Conclusion>

Table A shows these results. In the column of Introduction of GFP in Table A, "○" shows that the introduction of GFP into cells is confirmed, and "×" shows that the introduction of GFP into cells is not confirmed. In the column of Dead Cell in Table A, "○" shows that dead cells are not virtually confirmed, "△" shows that dead cells are confirmed and those dead cells account for 50% or less of all cells, and "×" shows that dead cells are confirmed and those dead cells account for more than 50% of all cells.

[Table A]

		Introduction of GFP	Dead Cell
Native Avidine	Fig.2	×	○
Avidine-PEI600	Fig.3	○	○
Avidine-PEI1200	Fig.4	○	○
Avidine-PEI1800	Fig.5	○	△
Avidine-PEI3000	Fig.6	○	△
Avidine-PEI5000	Fig.7	○	×

Accordingly, when the conjugates formed by using PEIs having Mn of 3000 or less (named as "PEI600", "PEI1200", "PEI1800" and "PEI3000") were used, the conjugates were introduced into cells and major parts of the cells were living. In contrast, when the conjugate formed by using PEI having Mn of 5000 (named as "PEI5000") was used, the conjugate was introduced into cells, but the ratio of dead cells was high. Thereby, it is proves that when the conjugate formed by using PEI having Mn of more than 3000 is used, the introduction efficiency decreases actually.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed this 6 th day of September , 2006

Junichiro Futami

Junichiro Futami